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ORIGINAL PAPER

Transport of Na^+ and K^+ by an antiporter-related subunit from the *Escherichia coli* NADH dehydrogenase I produced in *Saccharomyces cerevisiae*

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Abstract The NADH dehydrogenase I from *Escherichia coli* is a bacterial homolog of the mitochondrial complex I which translocates Na^+ rather than H^+. To elucidate the mechanism of Na^+ transport, the C-terminally truncated NuoL subunit ($NuoL_N$) which is related to Na^+/H^+ antiporters was expressed as a protein A fusion protein (ProtA–$NuoL_N$) in the yeast *Saccharomyces cerevisiae* which lacks an endogenous complex I. The fusion protein inserted into membranes from the endoplasmatic reticulum (ER), as confirmed by differential centrifugation and Western analysis. Membrane vesicles containing ProtA–$NuoL_N$ catalyzed the uptake of Na^+ and K^+ at rates which were significantly higher than uptake by the control vesicles under identical conditions, demonstrating that ProtA–$NuoL_N$ translocated Na^+ and K^+ independently from other complex I subunits. Na^+ transport by ProtA–$NuoL_N$ was inhibited by EIPA (5-(*N*-ethyl-*N*-isopropyl)-amiloride) which specifically reacts with Na^+/H^+ antiporters. The cation selectivity and function of the NuoL subunit as a transporter module of the NADH dehydrogenase complex is discussed.

Keywords Bacterial respiration · Membrane protein expression · NADH dehydrogenase · Na^+ transporter · Cation antiporter

Abbreviations
EIPA 5-(*N*-ethyl-*N*-isopropyl)-amiloride
TPP^+ Tetraphenylphosphonium

Introduction

Chemical energy provides the driving force for the generation of electrochemical gradients across biological membranes by respiratory chain complexes, while secondary transport systems couple these electrochemical gradients to the energy-dependent translocation of solutes required by the living cell (Mitchell 1961). The NADH:quinone oxidoreductase in bacteria (NDH I) or mitochondria (complex I) represents the largest respiratory complex in the oxidative phosphorylation system which couples the oxidation of NADH to the translocation of protons (or Na^+) across the bacterial or mitochondrial membrane (Hirst 2005; Brandt 2006). Recently, the high-resolution structure of the peripheral, NADH-oxidizing part of the bacterial NDH I was reported (Sazanov and Hinchliffe 2006), but the pathway of the coupling cations (H^+ or Na^+) through its membrane-embedded part still remains enigmatic. NDH I comprises 14 subunits (NuoA–NuoN) which are considered to represent the functional core of the larger complex I found in mitochondria (Gabaldon et al. 2005). In energy-conserving hydrogenases, only six out of the 14 Nuo subunits are

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present, namely homologs of subunits NuoBCDI in the so-called peripheral part and NuoH/NuoL in the membrane-embedded part of the enzyme. These energy-conserving hydrogenases are phylogenetically related to complex I and translocate H^+ (or Na^+) across the membrane (Hedderich and Forzi 2005). The NuoH/NuoL homologs are likely to represent the transporter module of energy-conserving hydrogenases, suggesting that subunits NuoH and L likewise function in H^+ or Na^+ translocation by NDH I.

We previously showed that NDH I from *Escherichia coli* and the related enterobacterium *Klebsiella pneumoniae* transports Na^+ rather than protons (Steuber et al. 2000; Gemperli et al. 2003; Vgenopoulou et al. 2006). A likely candidate for Na^+ transport through the membranous part of NDH I is the NuoL subunit (ND5 in the bovine complex I). NuoL comprises 14 transmembrane helices and exhibits high sequence similarity with subunits found in oligomeric cation/proton antiporter complexes termed Mrp or Mnh (Mathiesen and Hägerhäll 2002, 2003). These secondary transporters play a role in Na^+ resistance and Na^+ -dependent pH homeostasis in *Bacillus subtilis* and were shown to catalyze Na^+/H^+ antiport in *Staphylococcus aureus* (Hiramatsu et al. 1998; Ito et al. 1999, 2000; Kosono et al. 1999; Blanco-Rivero et al. 2005; Swartz et al. 2005). A C-terminally truncated version of the NuoL subunit ($NuoL_N$) from *E. coli* NDH I comprising 11 hydrophobic segments which are conserved between NuoL and the A subunit from oligomeric antiporters was shown to significantly increase the Na^+ uptake activity of native *E. coli* membrane vesicles (Steuber 2003). However, the $NuoL_N$ protein was toxic for the *E. coli* host cells, and the possibility remained that the observed Na^+ transport was not catalyzed by $NuoL_N$ but resulted from partially assembled NDH I containing $NuoL_N$ (Steuber 2003).

Here, we describe the production of $NuoL_N$ in the yeast *Saccharomyces cerevisiae*. *S. cerevisiae* does not possess a complex I-type NADH dehydrogenase and is therefore ideally suited to study the function of individual complex I subunits. We show that the proteinA–$NuoL_N$ fusion protein is inserted into intracellular membranes from *S. cerevisiae* in a transport-competent state, and investigate its catalytic properties.

Materials and methods

Strains and media

Escherichia coli K-12 strain DH5α (MBI Fermentas) served as host for the amplification of plasmids which conferred ampicillin resistance. Cloning and expression of the truncated *nuoL* gene was performed using *S. cerevisiae* strains SS328, MHY500 and the MHY552 (the genotypes are given in Table 1) which are all *ura3* deficient and allow selecting for plasmids harboring the *URA3* gene required for uracil synthesis. Standard protocols were followed for yeast manipulation (Guthrie and Fink 1991) and transformation using lithium acetate (Gietz et al. 1992). Cells were grown at 30°C in YPD (2% bacto-peptone, 1% yeast extract, 2% glucose) or in minimal medium (0.67% yeast nitrogen base with appropriate supplements) containing 2% glucose or galactose as carbon source. *S. cerevisiae* (SC)-ura agar plates contained 2% agar in minimal medium without uracil.

Plasmids

Plasmids pGREG506, pGREG536 (Jansen et al. 2005) and Yep(ProtA-TEV-002c) (Schenk et al. 2001) were used to construct vectors for the production of the C-terminally truncated NuoL subunit from *E. coli* NDH-1 comprising N-terminal protein A (ProtA) or hemagglutinin (HA) tags (Table 1). Plasmid pGREG506 was *EcoR*I–*Not*I digested and gel-purified. The coding sequence of *S. aureus* protein A followed by a TEV protease recognition site was PCR amplified using the plasmid YEp(ProtA-TEV-002c) as template and the primers 5′- CAAGGAGAAAAAACCCCGGATTCTAGAGCGGCCGCACTAGT-3′ (forward) and 5′- TATGACGTCGACGGTATCGATAAGCTTGATATCGAATTCCCCTGAAAATACAGGTTTTC 3′ (reverse). 5′-regions of the primers which are homologous to plasmid pGREG506 are underlined. Bases annealing to the *protA* sequence are shown in bold letters. The start codon of the gene encoding protein A is shown in italics. Wild-type *S. cerevisiae* SS328 yeast cells were co-transformed with the purified PCR product and linearized pGREG506. Yeast transformants were selected on SC-ura agar plates and tested for the production of protein A under control of the *GAL1* promoter to confirm in vivo homologous recombination between the plasmid backbone and the PCR product to form the plasmid pGREG5006. Plasmid pGREG5006 was retrieved from positive yeast transformants by standard methods. Insertion of the *nuoL* gene fragment from 10,390 to 11,497 bp of the *E. coli* genome encoding for amino acid residues 1–369 of subunit NuoL into pGREG5006 followed the same in vivo recombination strategy yielding the expression vector pNLt1 for the production of the C-terminally truncated NuoL subunit with the ProtA epitope and the TEV protease cleavage site attached to its N-terminus (electronic supplementary material, Fig. S1). The nuoL gene fragment was PCR amplified with the primers 5′-GAATTCGATATCAAGCTTATCGATACCGTCGACA***ATG*AACATGCTTGCCTTAACC**-3′ (forward) and 5′-GCGTGACATAACTAATTACATGACTCGAGGTCGAC**TTAACGCAGACCGCCCATCTTGAAGATGTT**-3′ (reverse). 5′-regions of the primers which are homologous to plasmid

Table 1 Strains and plasmids used in this study

	Genotype or relevant characteristics	Origin or reference
Strains		
E. coli DH5*a*	*supE44 ΔlacU169* (**Φ** *80lacZΔ*M15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Hanahan (1983)
Saccharomyces cerevisiae SS328	*MATα ade2–101 his3Δ200 lys2-801 ura3-52*	Vijayraghavan et al. (1989)
Saccharomyces cerevisiae MHY500	*MAT**a** his3Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1*	Chen et al. (1993)
Saccharomyces cerevisiae MHY552	*MATα his3Δ 200 leu2-3, 112 ura3-52 lys2-80 trp1-1 ubc6-Δ1::HIS3 ubc7::LEU2*	Chen et al. (1993)
Plasmids		
pGREG506	Derivative of pRS316; y*east GAL1* promotor, *CYC1* terminator; *URA3*; Ap^r	Jansen et al. (2005)
YEp(ProtA-TEV-002c)	Containing ProtA-TEV-*YBR002c* coding sequence	Schenk et al. (2001)
pGREG5006	Derivative of pGREG506 containing the *Staphylococcus aureus* ProtA epitope followed by a TEV protease cleavage site under control of the yeast *GAL1* promoter; Ap^r	This study
pGREG536	Derivative of *pGREG506* contains 7xHA epitope tag under control of the yeast *GAL1* promotor; *URA3*; Ap^r	Jansen et al. (2005)
pNLt1	*Ec nuo*L_N inserted into pGREG5006; Ap^r	This study
pNLt2	*Ec nuo*L_N inserted into pGREG536; Ap^r	This study
pNLt3	*Ec nuo*L_N with C-terminal His6 tag cloned into pNLt1; Ap^r	This study
pRS316	Yeast shuttle vector; pBluescript derivative; *URA3*; *CEN6 ARS4*; Ap^r	Sikorski and Hieter (1989)

Ap^r Ampicillin resistance, *Ec E. coli*, *ProtA* protein A epitope from *Staphylococcus aureus*; *HA tag* synthetic peptide corresponding to amino acid residues 98–106 of human influenza virus hemagglutinin (HA), *His6 tag* six histidine residues, *TEV* Tobacco envelope virus protease recognition cleavage site

pGREG5006 are underlined. Regions encoding for *nuoL*$_N$ are shown in bold letters. The start codon of *nuoL*$_N$ is shown in italics. Yeast cells were co-transformed with the purified PCR product and *Sal*I-linearized pGREG5006 to obtain plasmid pNLt1 (electronic supplementary material, Fig. S1). Yeast transformants were screened for galactose-induced production of ProtA–NuoL$_N$ by SDS-PAGE and immunostaining using peroxidase–anti-peroxidase soluble complex antibody (PAP, Sigma). By the same strategy, the PCR amplified *nuoL* gene fragment was inserted into pGREG536 yielding the expression vector pNLt2 for the production of the HA–NuoL$_N$ fusion protein which was detected with an anti-HA antibody and horseradish peroxidase conjugate (Sigma). A part of the *nuoL* gene fragment including a C-terminal His6 tag was PCR amplified with the primers 5′**TGGTAACCGCGGGTGTCTACC3′** (forward) and 5′CATGACTCGAGGTCGACTTAGTGAT GATGATGATGATGA**CGCAGACCGCCCATCTTGA AG**3′ (reverse). 5′-regions of the primers which are homologous to plasmid pGREG5006 are underlined. Regions encoding for *nuoL*$_N$ are shown in bold letters. MYH550 and MHY552 yeast cells were co-transformed with the purified PCR product and *Sac*II/*Xho*I-linearized pNLt1 which lacks the region encoding for ProtA–NuoL$_N$. Homologous recombination yielded the expression vector pNLt3 encoding for ProtA–NuoL$_N$ with a His6 tag attached to its C-terminus (electronic supplementary material, Fig. S1). All vector constructs were confirmed by DNA sequencing. The sequences of the cloned *nuoL* fragments were identical to *nuoL* (accession number NP-416781) found on the *E. coli* genome (Blattner et al. 1997). The calculated molecular masses of the fusion proteins are 50585.6 Da (HA–NuoL$_N$), 57022.0 Da (ProtA–NuoL$_N$) and 57844.8 Da (ProtA–NuoL$_N$–His). As negative control for expression and transport studies, *S. cerevisiae* SS328 was transformed with plasmid pRS316 (Sikorski and Hieter 1989) containing no insert. Attempts to produce the full-length NuoL protein in *S. cerevisiae* were not successful. We could not retrieve plasmids encoding for NuoL after co-transformation with linearized vector and the PCR-amplified *nuoL* gene (not shown), although this approach was successful with the truncated version of *nuoL* described above. Our failure to retrieve vectors for the expression of *nuoL* suggests that as observed previously using *E. coli* as expression host (Steuber 2003), the NuoL subunit is toxic for *S. cerevisiae* cells.

Expression of NuoL$_N$ and fractionation of membranes

Saccharomyces cerevisiae cells containing the various plasmids were grown in 50 ml minimal medium containing 2%

glucose to mid-log phase at 30°C, washed twice with sterile H_2O and transferred to 1 l minimal medium containing 2% galactose to induce the *GAL1* controlled expression of the $NuoL_N$ fusion protein. The cells were grown in galactose at 30°C for 12 h prior to harvesting. 10^{10} cells (20 g wet weight) were collected by centrifugation, washed twice in buffer (50 mM Tris/HCl, pH 8.0, 100 mM KCl, 1 mM $MgCl_2$, 5% glycerol), suspended in 10 ml buffer and stored at −80°C.

Cells (10 g wet weight) were thawed in ice-water for 30 min, washed twice in 50 mM Tris/HCl pH 8.0, 0.25 M sucrose containing 5 or 10 mM chloride salts of choline or potassium, and resuspended in 20 ml of the same buffer. Subsequent steps were performed at 4°C and in the presence of 0.1 mM diisopropylfluorophosphate (Sigma). The cell suspension was mixed with 20 ml glass beads (425–600 µm, Sigma) and disrupted in a bead mill (Retsch) for 15 min at a frequency of 1/30 s^{-1}. Centrifugation for 5 min at 1700×*g* removed glass beads and unbroken cells and yielded the crude extract in the supernatant. To localize the HA-tagged $NuoL_N$ fusion protein in cellular compartments of *S. cerevisiae*, the crude extract was subjected to two centrifugation steps at increasing velocity which results in the fractionation of organelles and different membrane compartments according to their sedimentation properties (Baker et al. 1988; Deshaies and Schekman 1989). The first centrifugation (20,000×*g*, 30 min) yielded membranes from the ER and the nuclear envelope in the pellet. Ultracentrifugation of the supernatant (250,000×*g*, 2 h) separated the plasma membrane in the supernatant from mitochondria in the pellet. Aliquots from different membrane fractions (0.2 mg protein) were separated by SDS-PAGE and immunostained for protein A or hemagglutinin epitopes fused to $NuoL_N$. Antibodies against the WBP1 component of the *N*-oligosaccharyl transferase complex from *S. cerevisiae* (te Heesen et al. 1993) or the porin from *Neurospora crassa* (Court et al. 1996) were used to identify membranes derived from the ER or mitochondria, respectively.

To follow Na^+ uptake by ProtA–$NuoL_N$, the crude extract was centrifuged at 20,000×*g* (30 min) to obtain ER vesicles in the pellet. The pellet was washed with the desired buffer representing the internal lumen of the vesicles in subsequent transport studies. The vesicles were collected by a second centrifugation (20,000×*g*, 30 min), resuspended in 1–1.5 ml buffer (25–32 mg protein ml^{-1}), shock frozen in liquid nitrogen and stored at −80°C until use.

Na^+ and K^+ transport

Uptake of Na^+ by ER membrane vesicles was followed by atomic absorption spectroscopy (Gemperli et al. 2002), modified as follows. Reaction mixtures contained in 0.4 ml at room temperature: ER vesicles (2 mg protein), 10 mM Tris/MES, pH 8.0, and as denoted 5 or10 mM choline chloride and 5 or 10 mM KCl. If indicated, 100 µM EIPA (5-(*N*-ethyl-*N*-isopropyl)amiloride) (in DMSO, 1% in assays) or 50 µM valinomycin (in DMSO, 1% in assays) was added. The reaction was started by adding 5 mM NaCl to the reaction mixture (final concentration). At timed intervals, 70 µl (0.35 mg protein) were applied to a 1 ml plastic syringe containing 0.6 ml Dowex 50 (K^+). The vesicles were immediately eluted with 0.8 ml deionized water. The eluate containing Na^+ entrapped in the vesicles was analyzed by atomic absorption spectroscopy. In the absence of vesicles, 5 mM Na^+ in assay buffer applied to Dowex columns was absorbed completely. For K^+ transport measurements, the internal lumen of the vesicles contained 5 or 10 mM NaCl and 5 or 10 mM choline chloride. The vesicles (2 mg protein) were resuspended in10 mM Tris/MES, pH 8.0 containing 5 or 10 mM NaCl and 5 or 10 mM choline chloride. K^+ transport was initiated by adding 10 mM KCl. The reaction was followed as described for Na^+ uptake, but using Dowex 50 (H^+). Blank values were obtained with vesicles in buffer without NaCl or KCl added. The influence of a transmembrane potential (inside positive) on K^+ uptake by ProtA–$NuoL_N$ was studied using the lipophilic cation tetraphenylphosphonium (TPP^+). ER vesicles were incubated with 0.1 mM tetraphenylphosphonium chloride (Aldrich) in 10 mM Tris/MES, pH 8.0 for 10 min prior to the start of the reaction. Chloride salts of K^+ (10 mM) and TPP^+ (0.1 or 10 mM; final concentrations) were added at $t = 0$, and the internal K^+ content of the vesicles was determined as described above. Without added NaCl and KCl, the buffers contained 100 µM K^+ and 70 µM Na^+.

Vesicle volume

The internal volume of the vesicles was determined using phosphate as impermeable anion (Laubinger 1987; Laubinger and Dimroth 1988). Cell rupture and preparation of ER vesicles were performed in 50 mM potassium phosphate, pH 8.0. The external phosphate was removed by gel filtration on a Sephadex G-75 column equilibrated with 10 mM Tris/HCl, pH 8.0. Fractions containing vesicles were combined and mixed with 1% SDS to solubilize the membranes. After 30 min, the amount of inorganic phosphate in one aliquot was determined according to (Ames and Dubin 1960). A second aliquot was analyzed for the total phosphate content which included organic phosphates from phospholipids (Ames and Dubin 1960). The determination of inorganic phosphate allowed calculating the internal volume of the vesicles. Subtracting the amount of inorganic phosphate from the total phosphate content gave the phos-

pholipid content of the vesicles. The internal volume was 45 ± 15 µl mg^{-1} phospholipid, or 11 ± 4 µl mg^{-1} protein. Comparable values were obtained with vesicles in the absence or presence of ProtA–$NuoL_N$, and with vesicles preloaded with 5 mM instead of 50 mM potassium phosphate. This experimentally determined vesicle volume is comparable to a calculated vesicle volume of 24 µl mg^{-1} phospholipid which is based on the assumption that the diameter of the vesicle is 150 nm, and that the head group of a phospholipid with a molecular mass of 700 Da covers a surface area of 0.7 nm^2. The calculation of the vesicle volume is described in the electronic supplementary material.

Cryo electron microscopy

The size and morphology of the ER vesicles used for transport measurements was studied by cryo electron microscopy. Three microliters of *S. cerevisiae* ER vesicles containing ProtA–$NuoL_N$ or control vesicles (50 µg protein, diluted 1:1 in buffer containing 50 mM Tris/HCl pH 8.0 and 10 mM choline chloride) were applied to glow-decharged Lacey carbon film (300 mesh Plano Wetzlar) within the environmental chamber (humidity 100%, 22°C) of the Vitrobot (Fei), followed by blotting and plunging into a liquid ethane bath. The grids were transferred to a Gatan cryoholder (Gatan, Warrendale, PA, USA) and kept at a temperature below −170 °C. Micrographs were recorded with a 2 k CCD camera at a magnification of ×53,000 using the low-dose procedure with a Philips TecnaiF20 electron microscope operated at 200 kV.

Enzyme immunoassays

The accessibility of the protein A tag fused to the N-terminus of $NuoL_N$ was determined from the chromogenic reaction of the peroxidase–anti-peroxidase conjugate antibody (PAP) using BM blue (Roche) as substrate. All steps were performed at room temperature. Vesicles before and after solubilization with detergent, and with and without ProtA–$NuoL_N$ were compared. ER vesicles in buffer (10 mM Tris/MES, pH 8.0, 10 mM choline chloride) were obtained from *S. cerevisiae* strain SS328 transformed with vector pNLt1 (ProtA–$NuoL_N$) or pRS316 (control). To aliquots of 15 µl (0.6 mg protein), 50 µl buffer with or without 4% (by weight) Triton X-100 was added. The vesicles were agitated for 1 h and mixed with 200 µl of a 1:5000 dilution of PAP. After 1 h, 40 µl ImmunoPure® Immobilized Protein A beads (Pierce) were added and allowed to react with unbound PAP for 30 min. The beads were removed by centrifugation at 5,000×*g* for 2 min to obtain PAP bound to ProtA–$NuoL_N$ in the supernatant. Enzyme assays for the detection of protein A-bound peroxidase were performed in microtiter plates (Maxisorb, Nunc) pretreated with 2.5% milk powder and 3% BSA (per weight) in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05 % Tween 20). To each well, 100 µl of the supernatant, 50 µl TBST, and 100 µl BM blue substrate were added. After 5 min, the peroxidase was inactivated by the addition of 50 µl 1 M H_2SO_4, and the concentration of the yellow reaction product was determined from the difference of absorption at 450 and 650 nm on a bio assay reader (Perkin Elmer HTS 7000 Plus). The values were corrected by subtracting the absorbance of a control without vesicles ($A_{450–650\ nm} = 0.120 \pm 0.001$). Mean values from three experiments are presented.

Analytical methods

Protein was determined by the bicinchoninic acid method (Pierce). BSA served as standard. SDS-PAGE was performed with 10% polyacrylamide as described in (Schägger and von Jagow 1987). Ten standard proteins in the range from 250 to 10 kDa were used (Precision Plus Protein Standards, Bio Rad). ProtA–$NuoL_N$ or HA–$NuoL_N$ was transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Bioscience) and immunostained with peroxidase–anti-peroxidase or anti-HA antibody and horseradish peroxidase conjugate (Sigma) by chemiluminescence (ECL, Amersham Bioscience).

Results

The $NuoL_N$ fusion proteins are inserted into membranes from the endoplasmatic reticulum

We produced the C-terminally truncated NuoL subunit from *E. coli* NDH I ($NuoL_N$) which was N-terminally fused to the hemagglutinin (HA) or protein A (ProtA) epitope (electronic supplementary information, Fig. S1) and studied the cellular localization of the fusion proteins in *S. cerevisiae*. Membranes from different cellular fractions were analyzed by SDS-PAGE followed by immunostaining using antibodies against the HA- or protein A epitopes. We confirmed that vesicles derived from the ER were separated from mitochondria by our fractionation method using antibodies specific for WBP1, a component of the *N*-oligosaccharyl transferase complex in the ER membrane (te Heesen et al. 1993), and for a porin from yeast mitochondria (Court et al. 1996) (Fig. 1). The HA–$NuoL_N$ fusion protein which exhibited an apparent mass of approximately 45 kDa was detected in whole cell lysates and in membranes derived from the endoplasmatic reticulum (ER) and the nuclear envelope, but not in fractions which predominantly contained mitochondria, microsomes, Golgi membranes or plasma membranes. In the latter fractions, only the HA-tag with an approximate mass of 10 kDa was present,

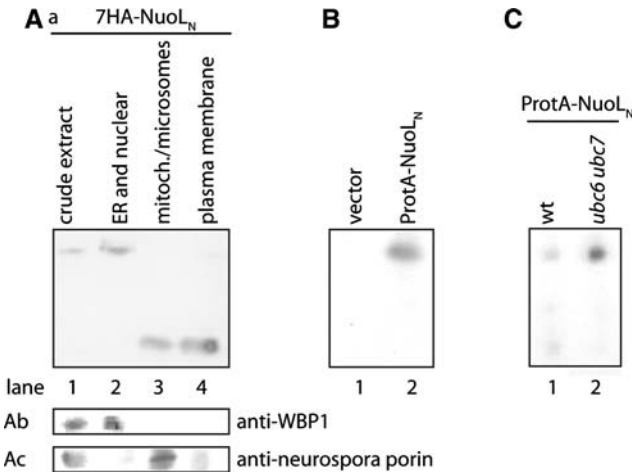

Fig. 1 Localization of NuoL$_N$ in subcellular compartments from *Saccharomyces cerevisiae*. The membranes (0.2 mg protein) were separated by SDS-PAGE, and NuoL$_N$ was detected by immunostaining using antibodies against the N-terminal tag of the NuoL$_N$ fusion proteins. Specific antibodies were used to detect the ER membrane protein WBP1 and the porin from the outer membrane of mitochondria. **a** *lane 1* crude cell lysate; *lane 2* endoplasmatic reticulum (ER) and nuclear membranes; *lane 3* microsomes and mitochondria; *lane 4* plasma membrane; *Aa* HA–NuoL$_N$; *Ab* WBP1, *Ac* porin. **b** ER membranes from *S. cerevisiae* SS328 transformed with pRS316 (*lane 1* negative control) or pNLt1 (*lane 2* ProtA–NuoL$_N$). **c** ER membranes from different *S. cerevisiae* strains transformed with pNLt3 producing His-tagged ProtA–NuoL$_N$; *lane 1 S. cerevisiae* strain MHY500; *lane 2 S. cerevisiae* strain MHY552 deficient in ubiquitylation *(Δubc6 Δubc7)*. Due to their hydrophobic nature, the NuoL$_N$ fusion proteins migrate faster on SDS-PAGE and exhibit apparent molecular masses of approximately 45 kDa. See Materials and methods for the calculated masses

indicating proteolytic degradation of HA–NuoL$_N$. Very similar results were obtained with the ProtA–NuoL$_N$ fusion protein which exhibited an apparent molecular mass of approximately 45 kDa and was predominantly found in membranes from the ER (Fig. 1b). All NuoL$_N$ fusion proteins investigated (N-terminally fused to HA or protein A, or with N-terminal protein A and C-terminal His-tag) migrated further in SDS- PAGE than anticipated from their calculated molecular masses, a phenomenon frequently observed with hydrophobic proteins. Please note that the detection of the isolated HA or protein A tags in membrane fractions does not indicate their membrane localization. We did not wash the membranes prior to Western blot analysis which would be necessary to remove these soluble proteins resulting from proteolytic degradation of the NuoL$_N$ fusion proteins.

The nature of the N-terminal fusion proteins did not influence the localization of NuoL$_N$ in ER vesicles from *S. cerevisiae*, suggesting that this experimental system will allow membrane insertion of NuoL$_N$ fused to other hydrophilic proteins, for example subunits from the peripheral part of complex I. The additional presence of a His-tag at the C-terminus of NuoL$_N$ was also tolerated (Fig. 1c), allowing purification of NuoL$_N$ by affinity chromatography in the future. Lysates from control cells transformed with empty vector (pRS316) did not contain proteins with cross-reactivity against antibodies specific for the NuoL$_N$ tags, as shown for the peroxidase–anti peroxidase conjugate detecting the protein A epitope (Fig. 1b).

The NuoL$_N$ fusion proteins were inserted into intracellular membranes without a specific leader sequence, as reported for other membrane proteins which were heterologously produced in *S. cerevisiae* (Griffith et al. 2003). We studied the orientation of ProtA–NuoL$_N$ in ER vesicles by comparing the accessibility of the protein A epitope before and after solubilization of the vesicles with Triton X-100. In the enzymatic immunoassay used, the absorbance of the reaction product was proportional to the amount of protein A exposed to the antibody conjugate. Vesicles without ProtA–NuoL$_N$ exhibited an absorbance $A_{450–650\ nm} = 0.154 \pm 0.033$ before, compared to 0.155 ± 0.030 after treatment with detergent. Vesicles containing ProtA–NuoL$_N$ exhibited an absorbance $A_{450–650\ nm} = 0.501 \pm 0.047$ in the absence of detergent which did not significantly increase after disruption of the vesicles with Triton X-100 ($A_{450–650\ nm} = 0.511 \pm 0.034$). We conclude that ProtA–NuoL$_N$ inserted into ER vesicles in a uniform manner, resulting in an orientation where the N-terminus of the protein was exposed to the external side of the vesicles.

We compared the stability of ProtA–NuoL$_N$ in a *S. cerevisiae* strain deficient in ubiquitylation *(Δubc6 Δubc7)* and the isogenic wild-type strain and observed decreased proteolytic degradation of ProtA–NuoL$_N$ in the *Δubc6 Δubc7* double mutant cell background (Fig. 1). Ubiquitylation of ProtA–NuoL$_N$ is a prerequisite for proteasome-mediated degradation after retro-translocation from the ER into the cytoplasm (Meusser et al. 2005). The diminished degradation observed in the *UBC6* and *UBC7*-deficient strain suggested that ProtA–NuoL$_N$ was proteolytically degraded by the proteasome. Proteolytic degradation of the NuoL$_N$ fusion proteins in the cytoplasm yielded the soluble protein A or hemagglutinin domains which were detected by immunostaining in different cellular fractions (Fig. 1).

We investigated whether the ER fraction from *S. cerevisiae* producing ProtA–NuoL$_N$ contained lipid vesicles suitable for transport studies. The size and morphology of the ER membranes containing ProtA–NuoL$_N$ were studied by cryo electron microscopy (Frederik and Hubert 2005). High salt concentrations during the isolation of the ER membranes were avoided because they are known to result in extensive breakdown of the nuclear envelope (Agutter 1972; Bornens and Courvalin 1978; Widmer and Parish 1980). Figure 2 shows the micrographs of ER membranes with and without ProtA–NuoL$_N$ embedded in vitrous ice. Both membrane preparations contained intact spherical vesicles with

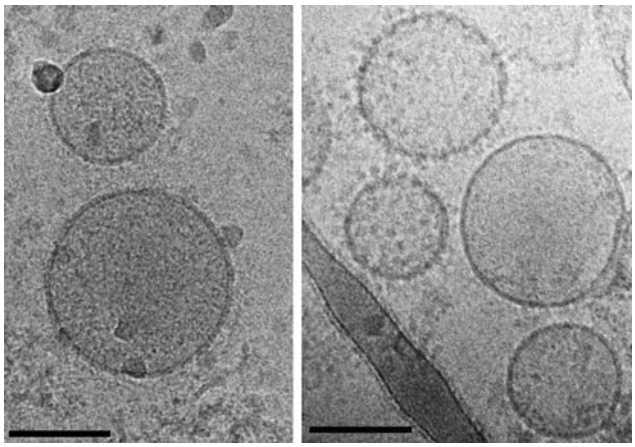

Fig. 2 Cryo electron microscopy of vesicles from *S. cerevisiae* used for transport studies. Micrographs of ER membranes from *S. cerevisiae* cells containing ProtA–$NuoL_N$ (*left panel*) and from control cells transformed with empty vector (pRS316 *right panel*) were recorded at a magnification of ×53,000. The *scale bar* indicates 100 nm

a diameter from 50 to 300 nm well suited to study cation transport. In addition, few open membrane sheets were observed. These membrane fragments do not interfere with Na^+ or K^+ uptake measurements since only cations entrapped in closed vesicles were analyzed (Gemperli et al. 2003). Since *S. cerevisiae* does not contain NADH dehydrogenases belonging to the complex I family of enzymes (Sickmann et al. 2003), we could study cation transport by ProtA–$NuoL_N$ into ER vesicles in the absence of other complex I subunits, as described in the following section.

Na^+ transport by ProtA–$NuoL_N$ in response to ΔpNa^+ and $\Delta\Psi$

First, we asked whether ProtA–$NuoL_N$ promotes Na^+ transport into the ER vesicles driven by the chemical Na^+ concentration gradient, ΔpNa^+. Membrane vesicles with or without ProtA–$NuoL_N$ were loaded with 10 mM KCl and then added to assay buffer containing 10 mM choline chloride and 100 µM K^+. Na^+ uptake was started by adding 5 mM NaCl to the external buffer at $t = -40$ s. We determined the internal Na^+ content of vesicles prior to the start of the reaction (1–4 nmol mg^{-1} protein) and calculated an internal Na^+ concentration of 0.09–0.36 mM which was based on the experimentally determined vesicle volume of 11 µl mg^{-1} protein. Within 20 s, the internal Na^+ content of vesicles containing ProtA–$NuoL_N$ increased to 47 nmol Na^+ mg^{-1} protein compared to 20 nmol Na^+ mg^{-1} protein in the control vesicles. By subtracting the amount of Na^+ accumulated in the control vesicles, we obtained a Na^+ uptake rate of ProtA–$NuoL_N$ of 81 ± 7 nmol min^{-1} mg^{-1} (Fig. 3). At $t = 0$, a transmembrane voltage $\Delta\Psi$ (negative inside) was applied by adding valinomycin which resulted in further Na^+ uptake by vesicles containing ProtA–$NuoL_N$ but had no effect on the control vesicles. After 100 s the internal Na^+ content of vesicles containing ProtA–$NuoL_N$ reached 80–100 nmol Na^+ mg^{-1} protein corresponding to an estimated Na^+ concentration in the vesicle lumen of 7–9 mM, compared to 20–30 nmol Na^+ mg^{-1} protein in the control vesicles. In the presence of $\Delta\Psi$ (negative inside), the activity of ProtA–$NuoL_N$ resulted in the accumulation of Na^+ in the lumen of the vesicles to concentrations which were higher than the external Na^+ concentrations. The observation that the control vesicles did not accumulate Na^+ in response to $\Delta\Psi$ (negative inside) suggested that there was only little unspecific leakage of Na^+ into ER vesicles from *S. cerevisiae*. We also compared Na^+ transport by ProtA–$NuoL_N$ in a *S. cerevisiae* strain deficient in ubiquitylation (*Δubc6 Δubc7*) and in the isogenic wild-type strain. In the wild-type strain, proteolytic degradation decreased the amount of ProtA–$NuoL_N$ (Fig. 1) and diminished Na^+ transport by 30% compared to the mutant strain (data not shown). In summary, these results unequivocally demonstrate that ProtA–$NuoL_N$ catalyses ΔpNa^+-driven Na^+ transport. The data confirm the previous notion that the C-terminally truncated NuoL subunit from *E. coli* complex I possesses Na^+ transport activity (Steuber 2003), and are consistent with our finding that complex I from *E. coli* has the capacity to translocate Na^+ (Steuber et al. 2000).

Transport of Na^+ by ProtA–$NuoL_N$ into the ER vesicles is expected to generate a transmembrane potential (inside positive) unless it is coupled to cation antiport or anion symport which would turn the overall transport process electroneutral. We used valinomycin to either create a transmembrane potential (inside negative) at low external $[K^+]$, or to dissipate the transmembrane voltage ($\Delta\Psi = 0$) at high external and internal K^+ concentrations ($[K^+]_{in} = [K^+]_{out} = 10$ mM). The reaction was started by adding 5 mM Na^+ at $t = -40$ s. Essentially identical rates of initial Na^+ transport by ProtA–$NuoL_N$ were observed at low and high external $[K^+]$ (0.14 µmol min^{-1} mg^{-1}). After 40 s, valinomycin was added, and Na^+ transport was followed for another 100 s. In the presence of $\Delta\Psi$ (inside negative), the Na^+ content of vesicles containing ProtA–$NuoL_N$ reached a final value of about 88 nmol mg^{-1} protein, compared to 45 nmol Na^+ mg^{-1} at $\Delta\Psi = 0$ (Fig. 3). These results indicated that Na^+ transport by ProtA–$NuoL_N$ was stimulated by a transmembrane potential (inside negative). Next, we wanted to investigate whether Na^+ transport by ProtA–$NuoL_N$ was inhibited by applying a transmembrane potential (positive inside) prior to the addition of Na^+. Using again K^+/valinomycin to generate $\Delta\Psi$, we first had to confirm Na^+ uptake by ProtA–$NuoL_N$ at low internal K^+ concentrations. Quite unexpected, Na^+ transport by ProtA–$NuoL_N$ was only observed if K^+ (≥10 mM) was present in the lumen of the vesicles. At an internal K^+ concentration

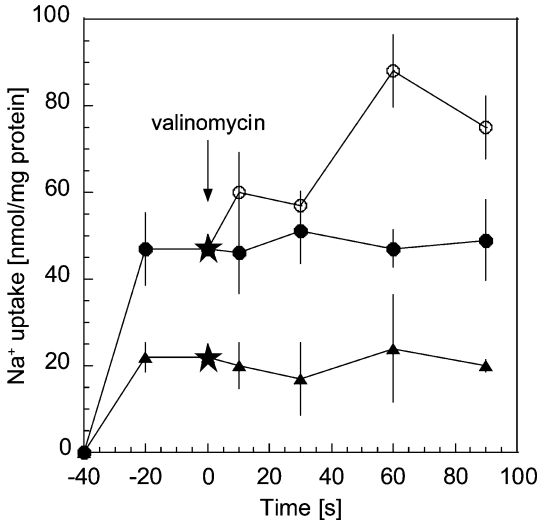

Fig. 3 $NuoL_N$ catalyzes Na^+ uptake into vesicles from *S. cerevisiae*. Na^+ uptake was started at $t = -40$ s by adding 5 mM NaCl to membrane vesicles (2 mg protein) prepared in 10 mM Tris/HCl pH 8.0, 10 mM KCl (internal lumen). The reaction was performed in 10 mM Tris/MES pH 8.0 containing 10 mM choline chloride or 10 mM KCl as denoted (external lumen of the vesicles). At $t = 0$, 50 µM valinomycin (in DMSO, final concentration 1%) was added (indicated by *stars*) to establish a transmembrane potential (negative inside) at external $[K^+] = 0.1$ mM (*open circles* and *closed triangles*) or to dissipate $\Delta\Psi$ at external $[K^+] = 10$ mM (*closed circles*). *Open circles*, vesicles with ProtA–$NuoL_N$ in 10 mM choline chloride; *closed circles*, vesicles with ProtA–$NuoL_N$ in 10 mM KCl; *closed triangles*, control in 10 mM choline chloride. Data points represent the average of six independent measurements corrected by the endogenous Na^+ content of the vesicles (1–4 nmol Na^+ mg^{-1} protein)

of 0.1 mM, the rates of Na^+ uptake and the final Na^+ content of vesicles did not differ significantly in vesicles with or without ProtA–$NuoL_N$ (Fig. 4). These results indicated that Na^+ transport by ProtA– $NuoL_N$ from the external to the internal aspect of the vesicle membrane was stimulated by K^+ present in the internal lumen.

K^+ transport by ProtA–$NuoL_N$

The observed stimulation of Na^+ translocation by K^+ could reflect a requirement of K^+ as a co-substrate during Na^+ transport by ProtA–$NuoL_N$. We studied K^+ transport by ProtA–$NuoL_N$ with vesicles containing 0.07, 5 or 10 mM Na^+ in the external and internal buffer. Within 10 s upon addition of K^+ to the external buffer, the internal K^+ content of vesicles containing ProtA–$NuoL_N$ increased significantly in the presence of 5 or 10 mM Na^+ compared to the control vesicles, while there was no significant K^+ uptake by ProtA–$NuoL_N$ vesicles at 0.07 mM Na^+ (Fig. 5). After 30 s, vesicles with ProtA–$NuoL_N$ contained 75 nmol K^+ mg^{-1} protein (10 mM Na^+), compared to 20 nmol K^+ mg^{-1} protein in the control vesicles under identical conditions, indicating that ProtA–$NuoL_N$ translocated K^+ into the vesicles driven by the K^+ concentration gradient. Since Na^+ transport by ProtA–$NuoL_N$ was stimulated by K^+ (Fig. 4), the results suggested that ProtA–$NuoL_N$ catalyzed the coupled translocation of Na^+ and K^+. The K^+ transport activity of ProtA–$NuoL_N$ may partially dissipate K^+ diffusion potentials established with valinomycin. One should therefore consider that a transmembrane potential generated with K^+/valinomycin is not constant during the course of a Na^+ uptake experiment with ProtA–$NuoL_N$ vesicles. K^+ transport into the control vesicles was essentially identical at the different Na^+ concentrations tested (Fig. 5), while Na^+ uptake by the controls was stimulated by K^+ to some extent (Fig. 4). We speculate that the endogenous Na^+ transporters present in ER vesicles from *S. cerevisiae* exhibit increased activity in the presence of K^+.

Next, we asked whether K^+ transport by ProtA–$NuoL_N$ was electrogenic and compared K^+ uptake by ProtA–$NuoL_N$ vesicles in the absence or presence of a transmembrane potential (inside positive) generated with the help of the lipophilic cation tetraphenylphosphonium (TPP^+). After

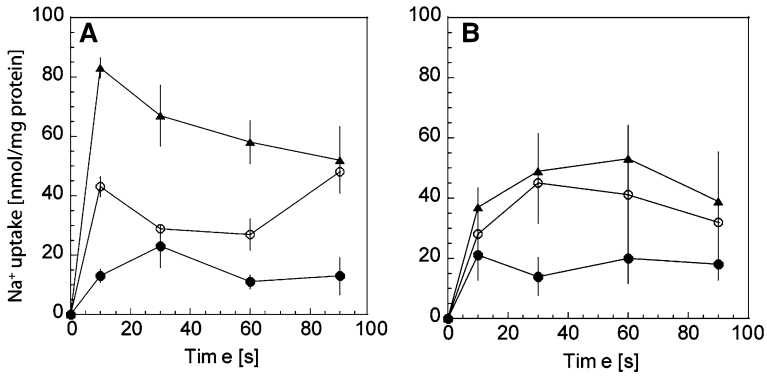

Fig. 4 Influence of K^+ on Na^+ uptake by $NuoL_N$. Vesicles were prepared from *S. cerevisiae* transformed with plasmid pNLt1 (ProtA–$NuoL_N$ **a**) or pRS316 (control **b**). The residual K^+ concentration of the internal and external buffer was 100 µM. Na^+ uptake was followed in 10 mM Tris/MES pH 8.0. The reaction was started by the addition of 5 mM NaCl at $t = 0$. The internal and external buffer contained 10 mM choline chloride (*closed circles*), 5 mM choline chloride and 5 mM KCl (*open circles*), or 10 mM KCl (*triangles*). Data points represent the average of three independent measurements and were corrected by the endogenous Na^+ content of the vesicles (1–4 nmol Na^+ mg^{-1} protein)

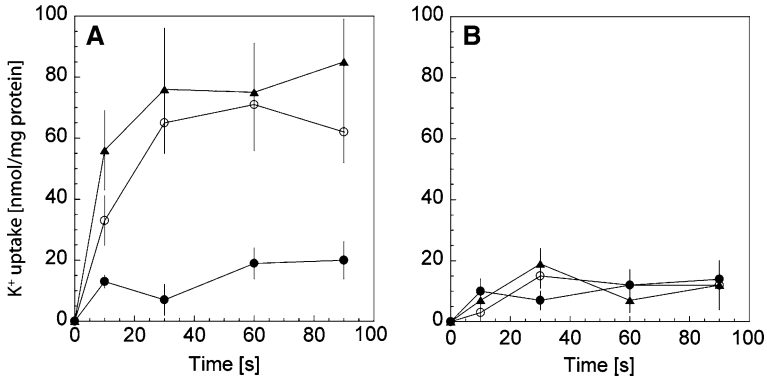

Fig. 5 Influence of Na^+ on K^+ uptake by $NuoL_N$. Vesicles were prepared from *S. cerevisiae* transformed with plasmid pNLt1 (ProtA–$NuoL_N$ **a**) or pRS316 (control **b**). The residual Na^+ concentration of the internal and external buffer was 70 μM. K^+ uptake was followed in 10 mM Tris/MES, pH 8.0. The reaction was started by the addition of 10 mM KCl at $t = 0$. The internal and external buffer contained 10 mM choline chloride (*closed circles*), 5 mM choline chloride and 5 mM NaCl (*open circles*), or 10 mM NaCl (*triangles*). Data points represent the average of 3–4 independent measurements and were corrected by the endogenous K^+ content of the vesicles (10–12 nmol K^+ mg^{-1} protein)

30 s, the K^+ content of vesicles containing ProtA–$NuoL_N$ reached 76 nmol mg^{-1} protein in the absence of ΔΨ, compared to 46 nmol Na^+ mg^{-1} in the presence of ΔΨ (inside positive) (Fig. 6). These results indicated that K^+ uptake by ProtA–$NuoL_N$ was inhibited by ΔΨ and further support the notion that the overall transport reaction catalyzed by ProtA–$NuoL_N$ results in the build-up of a transmembrane potential.

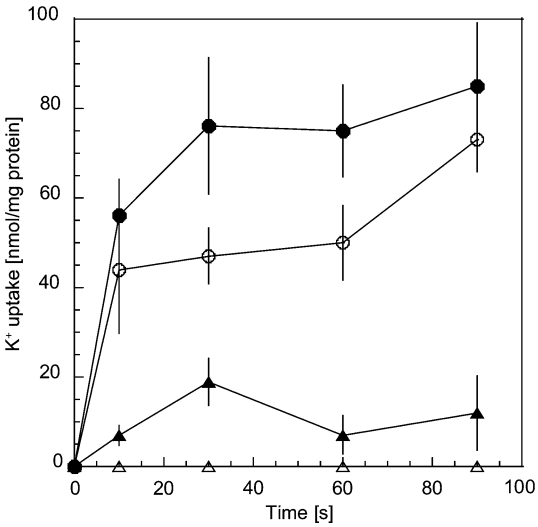

Fig. 6 Influence of a transmembrane voltage (inside positive) on K^+ transport by $NuoL_N$. Vesicles were prepared from *S. cerevisiae* transformed with plasmid pNLt1 (ProtA–$NuoL_N$ *circles*) or pRS316 (control; *triangles*). The external buffer (10 mM Tris/MES, pH 8.0) contained 10 mM NaCl. The reaction was started at $t = 0$ by adding 10 mM KCl and 0.1 mM tetraphenylphosphonium chloride (no transmembrane potential; *closed symbols*) or 10 mM KCl and 10 mM tetraphenylphosphonium chloride (ΔΨ inside positive; *open symbols*) to vesicles with 0.1 mM tetraphenylphosphonium chloride in the internal lumen. The data were corrected by the endogenous K^+ content of the vesicles (10–12 nmol K^+ mg^{-1} protein) and represent mean values from three experiments

Inhibition of Na^+ uptake by EIPA

5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) is a specific inhibitor of eukaryotic Na^+/H^+ antiporters but does not inhibit NhaA, the main Na^+/H^+ antiporter in *E. coli* (Padan and Schuldiner 1996). In the presence of 100 μM EIPA, Na^+ uptake by vesicles containing ProtA–$NuoL_N$ decreased by approximately 50% to rates observed with control vesicles (Fig. 7). Na^+ uptake by control vesicles (20 nmol Na^+ in the first 20 s after addition of NaCl) was not affected by EIPA (Fig. 7). Application of a transmembrane potential (negative inside) is expected to promote Na^+ uptake if the observed transport is due to unspecific leakage of Na^+ through the membranes. We found that the transmembrane voltage (inside negative) stimulated Na^+ transport by ProtA–$NuoL_N$, but did not increase Na^+ uptake by the control vesicles (Fig. 7). Most importantly, EIPA inhibited Na^+ transport by ProtA–$NuoL_N$ even in the presence of ΔΨ. The results indicated that the C-terminally truncated NuoL subunit from *E. coli* complex I exhibited higher sensitivity towards EIPA than the endogenous Na^+ transporter(s) present in the intracellular vesicles from *S. cerevisiae*. Na^+ uptake observed with control vesicles in the absence of ProtA–$NuoL_N$ was probably catalyzed by an intracellular Na^+/H^+ exchanger (NHE) like Nhx1 (Brett et al. 2005). Under our experimental conditions, EIPA specifically inhibited ProtA–$NuoL_N$ but not the other Na^+ transporter(s) present in the ER vesicles, suggesting that amilorides will be useful tools to probe cation transport by complex I subunits in native *S. cerevisiae* membranes.

Discussion

The functional analysis of NDH I is hampered by the lack of an expression system for the membrane-bound NuoH

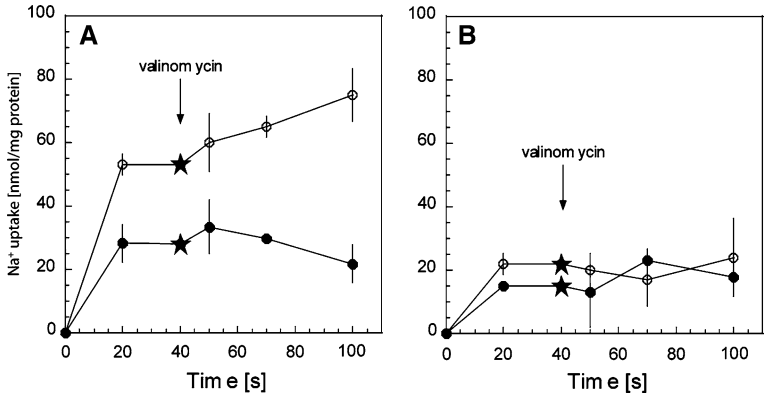

Fig. 7 EIPA inhibits Na^+ transport by $NuoL_N$. Na^+ transport was followed in 10 mM Tris/MES pH 8.0, 0.1 mM KCl, 10 mM choline chloride (external buffer) using ProtA–$NuoL_N$ vesicles (**a**) or control vesicles (**b**) loaded with 10 mM KCl. Vesicle aliquots (0.4 ml) were mixed with 4 µl DMSO or 100 µM EIPA (final concentration) from a 10 mM stock solution in DMSO. After 2 min, the reaction was started by adding 5 mM NaCl, and Na^+ uptake was followed in the absence (*open circles*) or presence of EIPA (*closed circles*). At $t = 40$ s, a transmembrane potential (negative inside) was established by adding 50 µM valinomycin (indicated by *stars*). Data points represent the average of 3–4 independent measurements and were corrected by the endogenous Na^+ content of the vesicles (1–4 nmol Na^+ mg^{-1} protein)

and NuoL subunits which are likely to play an important role during cation translocation. In this study, using *S. cerevisiae* as expression host, we demonstrate that the C-terminally truncated NuoL subunit ($NuoL_N$) of *E. coli* NDH I transports Na^+ and K^+ into native membrane vesicles in the absence of NADH. Since *S. cerevisiae* does not contain complex I, we conclude that the observed cation translocation is not catalyzed by a larger assembly of different complex I subunits containing the truncated NuoL subunit but in fact represents an intrinsic catalytic property of the $NuoL_N$ fusion protein. The experimentally determined topology for NuoL from *Rhodobacter capsulatus* complex I (Mathiesen and Hägerhäll 2002) would predict that the full-length NuoL subunit from *E. coli* complex I has 14 transmembrane helices, with its N-terminus located in the periplasm of the bacterial cell. In our vesicles derived from the ER of *S. cerevisiae*, ProtA–$NuoL_N$ was inserted in a uniform orientation, with the N-terminally fused protein A epitope exposed to the external side. These vesicles are therefore ideally suited to study the mode of cation transport by $NuoL_N$ in the future.

Over the last years, we accumulated evidence that NDH I from enterobacteria like *Klebsiella pneumoniae* (Gemperli et al. 2002, 2003) or *E. coli* (Steuber et al. 2000) translocates Na^+ rather than protons. Our views have been challenged by the hypothesis that complex I from *K. pneumoniae* (Bertsova and Bogachev 2004) or *E. coli* (Stolpe and Friedrich 2004) exclusively acts as a proton pump. However, we recently confirmed that under physiological conditions, e.g. at neutral or slightly alkaline pH and in the presence of 5 mM Na^+, complex I from *K. pneumoniae* pumps sodium ions (Vgenopoulou et al. 2006). The finding that the truncated NuoL subunit catalyzes Na^+ transport further corroborates our hypothesis that the enterobacterial NDH I preferentially uses Na^+ as a coupling cation. At present, we cannot exclude the possibility that in the absence of Na^+ and at acidic pH, e.g. under non-physiological conditions, NDH I may switch its cation specificity and translocate protons, as exemplified for the Na^+-translocating F1F0 ATPase from *Propionigenium modestum* (Kaim et al. 1997). There is evidence that the capacity to transport Na^+ is not restricted to complex I from enterobacteria. Hägerhäll and coworkers reported the complementation of a Na^+-sensitive *Bacillus subtilis* mutant with a plasmid encoding for subunit NuoL of complex I (NDH I) from *Rhodobacter capsulatus*. The presence of NuoL increased the Na^+ tolerance of the mutant strain, indicating that the isolated NuoL subunit from *R. capsulatus* complex I has Na^+ translocation ability in vivo (C. Hägerhäll, Lund University, personal communication).

Of the seven hydrophobic subunits comprising the membrane domain of NDH I, NuoL, NuoM and NuoN show sequence homology to subunit A from multicomponent cation/proton antiporters (Hamamoto et al. 1994; Mathiesen and Hägerhäll 2002, 2003). This evolutionary relationship makes NuoL a prime candidate for a Na^+ (or proton) channel through NDH I. From the sequence similarity of NuoL with multisubunit [Na^+ or K^+]/H^+ antiporters (Putnoky et al. 1998; Swartz et al. 2005), we consider the transport of H^+ and/or K^+ and/or Na^+ by $NuoL_N$. The shortened version of NuoL used in this study comprises 11 hydrophobic helixes and includes the part of the protein which is mostly related to the cation/H^+ antiporter A subunit. Notably, Na^+ uptake by $NuoL_N$ into ER membrane vesicles was stimulated by K^+, while K^+ transport by $NuoL_N$ required Na^+. The data are in accordance with a coupled transport of Na^+ and K^+ by $NuoL_N$. Further experiments are required to investigate whether protons are also transported, and to

determine the mode and stoichiometries of cation translocation by $NuoL_N$. The observation that uptake of Na^+ or K^+ was influenced by an applied transmembrane voltage indicates that the overall transport reaction catalyzed by $NuoL_N$ is electrogenic.

The finding that Na^+ transport by $NuoL_N$ was not dependent on NADH is in marked contrast to the situation in the holo-NDH I. Here, Na^+ translocation was strictly coupled to the oxidation of NADH even in the presence of a chemical Na^+ gradient which would have favored uptake of Na^+ by the proteoliposomes (Steuber et al. 2000; Gemperli et al. 2002, 2003). Obviously, Na^+ transport through the NuoL subunit in the holo-NDH I is prevented, most likely by the interaction of NuoL with other components of the complex. Candidates are (1) the C-terminal part of NuoL which is not included in our fusion protein, (2) other membrane-bound subunits, or (3) the peripheral, NADH-oxidizing fragment which could block the access of the coupling cation to Na^+ binding site(s) in the membranous arm of complex I. At present, it is unclear how NADH-dependent electron transfer initiated at the peripheral arm of NDH I provides the driving force for the endergonic transport of Na^+ through NuoL. Although a requirement of cofactors like quinones for Na^+ translocation by ProtA–$NuoL_N$ cannot be unequivocally excluded yet, it should be stressed that transport by the truncated NuoL subunit was observed without added electron donor. We therefore conclude that transport of Na^+ (or K^+) is not necessarily coupled to an electron transfer reaction of a putative redox cofactor located in $NuoL_N$. Na^+ transport by the truncated NuoL subunit from *E. coli* complex I was inhibited by EIPA, an amiloride derivative which is specific for mammalian Na^+/H^+ antiporters but only moderately affects NhaA, the main Na^+/H^+ antiporter in *E. coli* (Padan and Schuldiner 1996). In agreement with our results, Yagi and coworkers showed that NADH oxidation by complex I from *E. coli* was inhibited by EIPA and other amilorides (Nakamaru-Ogiso et al. 2003b). EIPA also prevented labeling of the ND5 subunit (NuoL homolog) of bovine complex I with a photoaffinity analogue of fenpyroximate, a potent complex I inhibitor which binds at or close to a quinone binding site (Nakamaru-Ogiso et al. 2003a). To analyze energy coupling by *E. coli* complex I in membrane vesicles, Yagi and colleagues used the complex I-specific substrate deaminoNADH (Kao et al. 2005a; Kao et al. 2005b). Electron transfer via complex I was coupled to the generation of a transmembrane voltage ($\Delta\Psi$) and a proton concentration gradient (ΔpH), and both components of the proton motive force were dissipated by adding a protonophore. The formation of ΔpH during deaminoNADH oxidation is no proof for vectorial proton transport by complex I. The electron transfer reaction catalyzed by complex I results in the net consumption of one H^+ per deaminoNADH oxidized for the formation of quinol (QH_2) from quinone (Q). If Q like deaminoNADH binds at the external side of the *E. coli* vesicles, electron transfer will be accompanied by an alkalization of the external lumen. The finding that the transmembrane voltage generated during deaminoNADH oxidation by *E. coli* vesicles was dissipated by a protonophore was taken as further evidence for proton transport by complex I (Kao et al. 2005a, b). This conclusion is based on the assumption that protonophores should not affect $\Delta\Psi$ generated by a Na^+ pump, However, Bogachev and colleagues showed that electrophoretic proton movements in the presence of protonophores dissipated the $\Delta\Psi$ established by the Na^+ -translocating NADH:quinone reductase from *Vibrio alginolyticus* (Bogachev et al. 1997). The observed formation of ΔpH and $\Delta\Psi$ during deaminoNADH oxidation, and dissipation of $\Delta\Psi$ by a protonophore (Kao et al. 2005a, b), therefore do not exclude Na^+ transport by the *E. coli* complex I.

In summary, our study demonstrates that transport of Na^+ and K^+ by the truncated NuoL subunit from *E. coli* NDH I does not require other complex I subunits, and is not dependent on NADH-driven electron transfer reactions. The energy-conserving hydrogenases, complex I and the multimeric antiporters are phylogenetically related and may translocate Na^+, K^+ or H^+ by a common mechanism. The electron input module of the respiratory complexes can be a hydrogenase or an NADH dehydrogenase which could act in concert with a cation transporter module including the NuoL subunit or its homolog.

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